Developmental Cell, Vol. 5, 547–558, October, 2003, Copyright ©2003 by Cell Press

A Notch/Delta-Dependent Relay Mechanism Establishes Anterior-Posterior Polarity in *Drosophila*

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Summary

The anterior-posterior axis of *Drosophila* becomes polarized early in oogenesis, when the oocyte moves to the posterior of the germline cyst because it preferentially adheres to posterior follicle cells. The source of this asymmetry is unclear, however, since anterior and posterior follicle cells are equivalent until midoogenesis, when Gurken signaling from the oocyte induces posterior fate. Here, we show that asymmetry arises because each cyst polarizes the next cyst through a series of posterior to anterior inductions. Delta signaling from the older cyst induces the anterior polar follicle cells, the anterior polar cells signal through the JAK/STAT pathway to induce the formation of the stalk between adjacent cysts, and the stalk polarizes the younger anterior cyst by inducing the shape change and preferential adhesion that position the oocyte at the posterior. The anterior-posterior axis is therefore established by a relay mechanism, which propagates polarity from one cyst to the next.

Introduction

The ovary of *Drosophila* is composed of about 16–20 ovarioles, each of which contains a series of egg chambers that proceed through the 14 stages of oogenesis as they move from the anterior germarium toward the oviduct at the posterior (Spradling, 1993). The germline stem cells reside at the anterior tip of the germarium and divide asymmetrically to produce a new stem cell and a cystoblast, which then undergoes four consecutive mitoses with incomplete cytokinesis to give rise to a cyst of 16 interconnected germ cells. One of these cells is selected to become the oocyte and moves to the posterior of the cyst in region 3 of the germarium (de Cuevas and Spradling, 1998; Huynh and St Johnston, 2000). This asymmetric arrangement of the germ cells generates the first anterior-posterior (A-P) polarity in development and leads to the polarization of the A-P axis of the embryo through two signaling events between the oocyte and the somatic follicle cells. At stage 6, Gurken signals from the oocyte to induce the adjacent follicle cells to adopt a posterior rather than an anterior fate (González-Reyes et al., 1995; Roth et al., 1995). The posterior cells then send an unknown signal back to the oocyte at stage 7 to induce the formation of a polarized microtubule cytoskeleton, which directs the transport of *bicoid* mRNA to the anterior of the oocyte and of *oskar* mRNA to the posterior (Clark et al., 1994, 1997; Ruohola et al., 1991; Theurkauf et al., 1992). The localization of these transcripts defines the A-P axis of the embryo, since *bicoid* mRNA encodes the anterior morphogen that patterns the head and thorax of the embryo, and *oskar* mRNA defines the site of formation of the pole plasm, which contains the abdominal and germline determinants (Driever, 1993; Ephrussi and Lehmann, 1992).

Mutants that disrupt the movement of the oocyte to the posterior of the cyst give rise to bipolar egg chambers with symmetric oocytes that localize *bicoid* mRNA to both poles and *oskar* mRNA to the center, indicating that all subsequent anterior-posterior asymmetries depend on the positioning of the oocyte (González-Reyes and St Johnston, 1994). This morphogenetic movement occurs as the cyst moves from region 2b to 3 of the germarium. The cyst flattens to form a lens-shaped disc in region 2b, and somatic follicle cells migrate to separate the cyst from the preceding older egg chamber. As the cyst enters region 3, it rounds up with the oocyte at the posterior and eventually protruding into the surrounding follicle cell layer (González-Reyes and St Johnston, 1998a). This process requires the preferential adhesion of the oocyte to the follicle cells that surround the posterior of the cyst (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998a). Both the oocyte and these follicle cells independently upregulate the homophilic adhesion molecule *D*E-cadherin, and removal of *D*E-cadherin from either cell disrupts oocyte positioning. This has led to a model in which the upregulation of *D*E-cadherin in the oocyte allows it to outcompete the nurse cells for adhesion to the posterior follicle cells, thereby anchoring it at the posterior, as the cyst changes shape. Thus, the first cue for anterior-posterior polarity is the increased adhesiveness of the posterior follicle cells, although it is not known why these cells behave differently from the other follicle cells.

The follicle stem cells reside in region 2b of the germarium and give rise to two distinct lineages: the epithelial follicle cell precursors, which proliferate until stage 6 to generate most of the cells that surround each cyst, and the polar/stalk precursors (Margolis and Spradling, 1995; Tworoger et al., 1999). The latter exit mitosis at stage 1 to 2 of oogenesis and give rise to the symmetric pairs of polar cells at the anterior and posterior poles of the cyst and to the stalk that separates each cyst from the adjacent one. *Delta* mutant germline clones and *Notch* follicle cell clones fail to form polar cells, indicating that Delta signals from the germline to activate the Notch receptor in the polar/stalk precursors to induce them to adopt the polar cell fate (López-Schier and St Johnston, 2001). This induction requires *fringe*, which is upregulated in the polar/stalk precursors and

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renders them competent to respond to the Delta signal (Grammont and Irvine, 2001). Once the polar cells are specified, they express Unpaired, the ligand for the JAK/STAT pathway, and the resultant activation of JAK/STAT signaling plays two key roles in patterning the rest of the follicle cells. First, the polar cells induce uncommitted polar/stalk cell precursors to become stalk cells (McGregor et al., 2002). Overexpression of Unpaired causes all polar/stalk cell precursors to differentiate as stalk, whereas loss-of-function mutations in *hopscotch* (JAK) or *STAT92E* cause a loss of the stalk (Baksa et al., 2002; McGregor et al., 2002). Second, Unpaired signaling from the polar cells induces the adjacent epithelial follicle cells at each pole of the egg chamber to adopt a terminal fate (Xi et al., 2003). This induction is essential for axis formation because only the terminal cells are competent to respond to Gurken by becoming posterior. Unpaired also acts as a morphogen to specify three distinct terminal cell types at the anterior: the border cells, the stretched follicle cells, and the centripetal cells (Beccari et al., 2002; Silver and Montell, 2001; Xi et al., 2003). In the absence of Gurken signaling, all three cell types also form at the posterior of the egg chamber, indicating that the graded activity of JAK/STAT pathway creates a symmetric prepattern at both poles (González-Reyes and St Johnston, 1998b).

The analysis of follicle cell patterning raises an intriguing paradox. On the one hand, the anterior-posterior polarity of the follicle cell layer depends on the positioning of the oocyte, since this determines the direction of the Gurken signaling that makes the posterior cells different from the anterior ones. On the other hand, the positioning of the oocyte depends on the fact that oocyte adheres more strongly to the posterior follicle cells than to the other follicle cells, indicating that these posterior cells must already be different before the oocyte is positioned. Although the follicle cells that adhere to the oocyte have not been unambiguously identified, it has recently been proposed that they correspond to the posterior polar cells (Grammont and Irvine, 2002). In order to investigate the source of this early asymmetry, we have analyzed the function of the polar cells by disrupting Delta signaling from single germline cysts. Our results resolve this paradox by showing that the anterior and posterior polar cells are not equivalent in the germarium. However, the positioning of the oocyte does not depend on the posterior polar cells but on the anterior polar cells of the adjacent older cyst and the stalk that they induce. This leads us to propose a novel model for anterior-posterior axis formation in *Drosophila*, in which each cyst transmits polarity to the adjacent younger cyst.

Results

Delta Germline Clones Fuse with the Anterior Egg Chamber and Induce the Mispositioning of Its Oocyte

To investigate the role of the Notch pathway in specifying the polar and stalk cell fate, we used the hsFLP-FRT technique to generate germline clones of a loss-of-function mutation in *Delta* (Dl^{M1}) that are marked by the absence of nlsGFP (Chou and Perrimon, 1996; Luschnig et al., 2000). As previously described, *Delta* germline clones lead to the formation of compound egg chambers, in which multiple germline cysts are surrounded by a common set of epithelial follicle cells (López-Schier and St Johnston, 2001). It is difficult to analyze follicle cells fates in the large compound egg chambers caused by germline stem cell clones, and we therefore concentrated on clones in which the mitotic recombination event produces a homozygous mutant cystoblast, which gives rise to a single mutant germline cyst flanked by wild-type cysts. These single cyst clones produce a penetrant and highly polarized phenotype, in which the mutant cyst fuses either completely or partially with the adjacent anterior cyst (183/200). In contrast, mutant cysts never fuse with the adjacent posterior cyst (Figures 1B and 1C). To confirm these results, we generated mitotic clones for a null allele of *Notch* (N^{55e11}) and looked for follicle cell clones that completely surrounded the cyst. As in *Delta* germline clones, these egg chambers fuse completely or partially to the adjacent anterior cyst but not to the posterior one (Figures 1D and 1E).

The oocytes of the wild-type anterior cysts fused to *Delta* mutant cysts are frequently mispositioned. The oocyte is most often localized to the posterior lateral side of the cyst, but it is also found at the anterior (Figures 1B and 1C). The same phenotype occurs in wild-type cysts fused to cysts with large follicle cell clones mutant for *Notch* (Figure 1D). As a consequence of its mispositioning, the oocyte of the anterior wild-type cyst develops a reversed anterior-posterior axis, in which components of the *oskar* mRNA localization complex, such as Staufen, localize to the anterior cortex of the oocyte, rather than the posterior (Figure 1D).

Each Egg Chamber Induces the Formation of Its Own Polar Cells

The asymmetry of the egg chamber fusions in *Delta* mutant cysts raised the possibility that Delta signaling is polarized toward the anterior. Since Delta signals from the germline to induce the formation of the polar cells, we examined which polar cells are lost when a single cyst is mutant for *Delta*. The anterior and posterior polar cells in wild-type egg chambers can be recognized by their expression of the polar cell specific enhancer trap lines A101 and PZ80, the presence of higher levels of FasIII and Myosin VI, and the lack of Eyes Absent protein, which is expressed in all follicle cells except the stalk and polar cells (Figure 2A). No polar cells ever form at the posterior of the mutant cysts, as assayed by all of these markers (Figures 2B–2F and data not shown). Instead, all of the follicle cells around a *Delta* mutant cyst have smaller nuclei and upregulate FasIII, because they continue dividing and are arrested in the precursor state (Figure 2B′; López-Schier and St Johnston, 2001). Each compound egg chamber does contain two sets of polar cells, however: an anterior pair that clearly associates with the anterior of the wild-type cyst and a second set that lies at the junction between the wild-type and mutant cysts (Figures 2C–2F). The latter cells contact the germ cells of the wild-type cyst in the cases of partial fusion, suggesting that they correspond to the posterior polar cells of the wild-type cyst and not the anterior

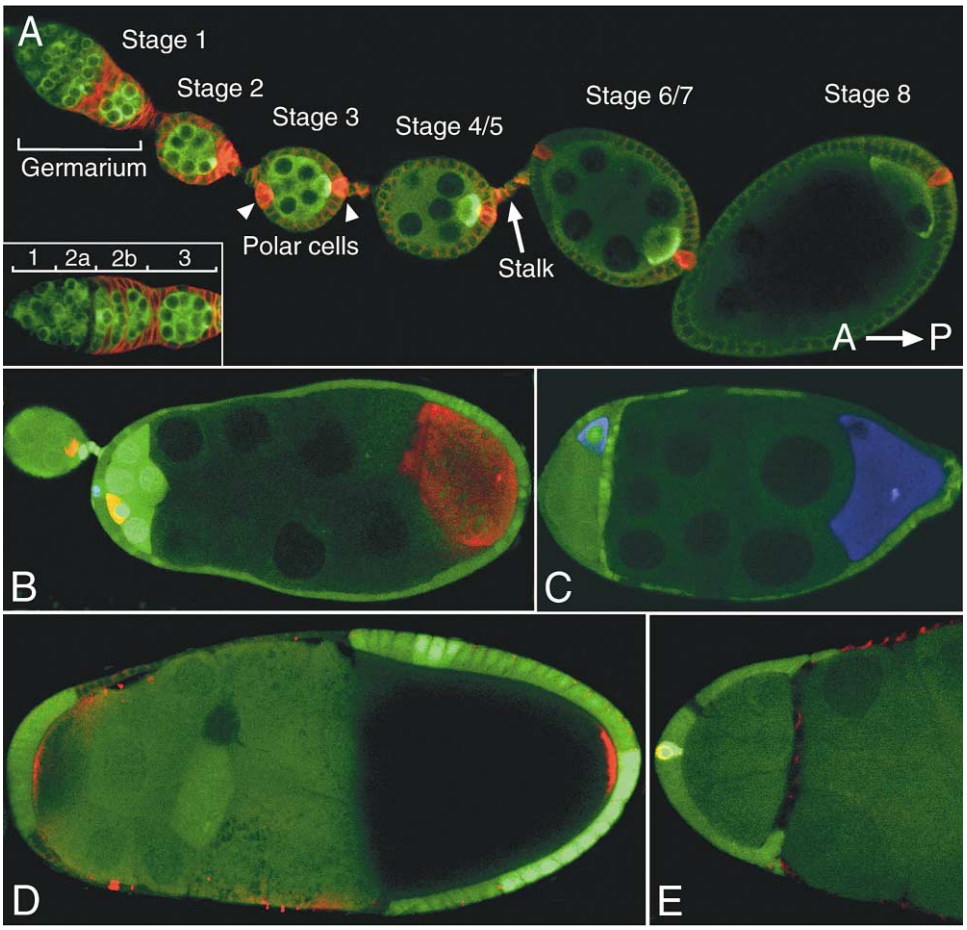

Figure 1. *Delta* Mutant Cysts Fuse with the Adjacent Anterior Wild-Type Egg Chambers

(A) Wild-type ovariole stained with anti-FasIII (red) and anti-BicD (green), which marks the oocyte. FasIII is expressed in all follicle cells until stage 3 when it becomes restricted to the polar cells. The box shows a magnified view of the germarium.

(B) A single *Delta* germline mutant cyst, marked by the absence of GFP (green), completely fused to the adjacent anterior wild-type cyst, which has its oocyte mispositioned to the anterior. The ovary has been stained with anti-β-gal (blue) to identify the polar cells, which express the PZ80 enhancer trap line, and with anti-Orb (red) to label the oocyte.

(C) A single *Delta* mutant cyst partially fused to the anterior wild-type egg chamber, which has the oocyte mislocalized to the posterior/lateral corner. The oocytes are labeled with anti-Orb (blue).

(D) A fusion between two egg chambers caused by a $Notch^{55e11}$ follicle cell clone, which is marked by the absence of nuclear GFP (green). The egg chambers have been stained with anti-Staufen (red) to mark the site of *oskar* mRNA localization at the posterior of the oocyte. The oocyte of the posterior cyst has a normal polarity, whereas the misplaced oocyte of the anterior cyst has a reversed polarity.

(E) Detail of a partial fusion between two egg chambers caused by a *Notch* follicle cell clone and stained for FasIII (red). All of the mutant cells continue to express FasIII, which is only expressed in polar cells at this stage in the wild-type situation.

polar cells of the mutant cyst (Figures 2D and 2F). To verify this, we analyzed compound egg chambers that contain one wild-type cyst and two or more consecutive *Delta* mutant cysts. In all cases, the egg chamber contains only two sets of polar cells at each end of the wild-type cyst and none at either end of the more posterior mutant cyst (Figure 2F). Thus, each germline cyst induces its own anterior and posterior polar cells.

The Anterior and Posterior Polar Cells Differentiate Asynchronously

Although the experiments above demonstrate that Delta signals symmetrically to specify the anterior and posterior polar cells, we observed that the anterior polar cells differentiate significantly earlier than the posterior ones. A101 is first expressed in the anterior polar cells at stage 1, just as the stalk begins to form to separate the cyst from the more anterior one (Figure 3A). This asymmetry persists as the cyst buds from the germarium, when A101 is expressed in four or sometimes more cells at the anterior of the cyst but not at the posterior (Figure 3B). At this stage, FasIII is still detectable in all follicle cells but is clearly enriched in the anterior cells. The first sign of posterior polar cell differentiation occurs at stage 2, when weak A101 expression and higher levels of FasIII can be detected in two or rarely three posterior cells (Figure 3C). By this stage, A101 is restricted to a pair of anterior polar cells that express high levels of FasIII and have a rounder shape than the neighboring follicle cells (Figure 3C″, arrow). Thus, the anterior polar cells differentiate at stage 1, about 12 hr before the posterior polar cells, which arise at stage 2. An identical asynchrony is

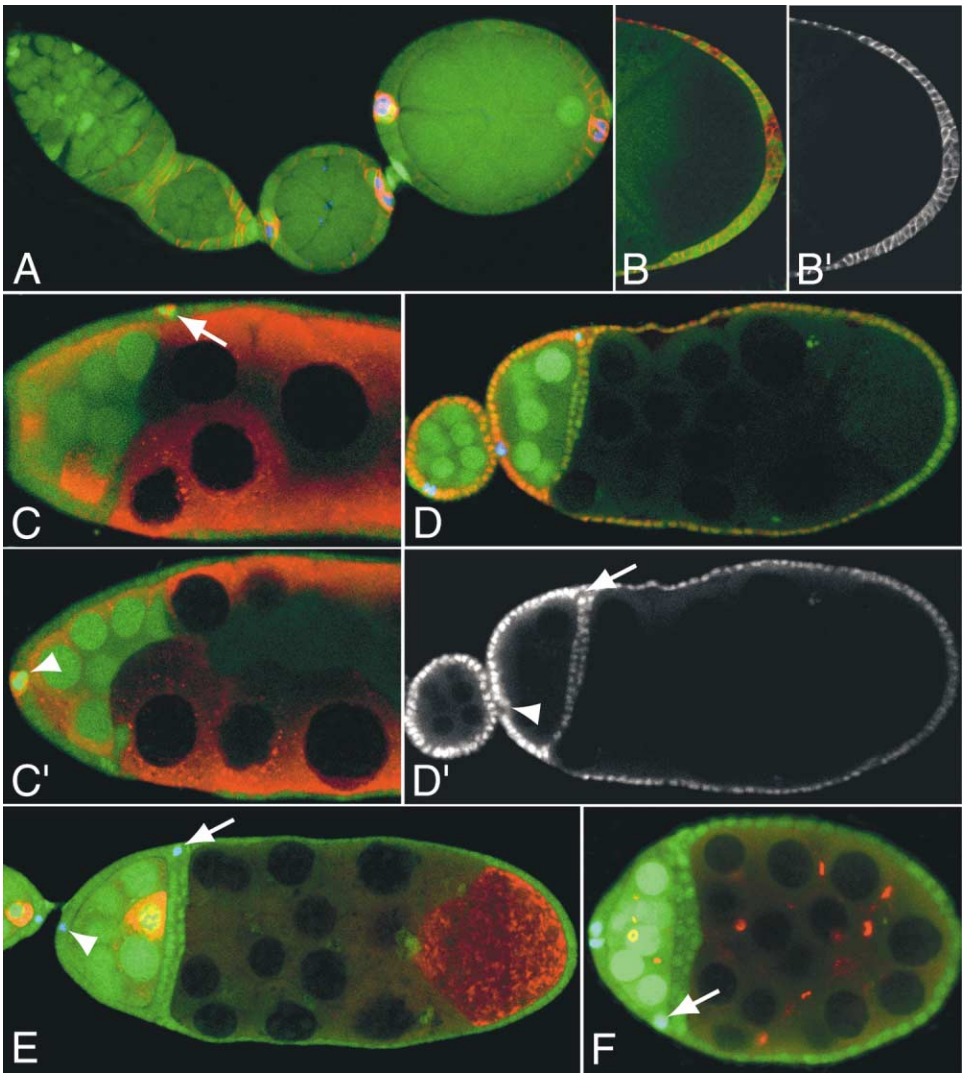

Figure 2. Each Egg Chamber Induces the Formation of Its Own Polar Cells

(A) Wild-type ovariole expressing the polar cell enhancer trap line A101 (blue) and stained with anti-FasIII (red). A101 is expressed in the pairs of polar cells at each pole of the egg chambers. FasIII is expressed in all follicle cells in the germarium but becomes restricted to the polar cells at later stages.

(B and B′) Detail of the posterior of a *Delta* germline mutant cyst stained for A101 (blue) and FasIII (red, B′). The egg chamber does not have posterior polar cells, as shown by the lack of A101 expression, but all of the follicle cells express FasIII because they are arrested at a precursor stage.

(C and C′) Two focal planes of a *Delta* germline clone (marked by the absence of GFP in green) that has completely fused to the anterior wild-type egg chamber. Myosin VI (red) is upregulated in the polar follicle cells. The wild-type egg chamber anterior to a mutant cyst has both posterior (C, arrow) and anterior (C′, arrowhead) pairs of polar cells.

(D and D′) The expression of A101 (blue) and Eyes Absent (red, D′) in a partial fusion between a wild-type egg chamber (anterior) and a *Delta* germline clone (posterior). The wild-type egg chamber has both anterior (arrowhead) and posterior (arrow) polar cells that can be distinguished by A101 expression and the lack of Eyes Absent, which is expressed in all follicle cells except the stalk and polar cells.

(E) The expression of A101 (blue) and Orb (red) in a partial fusion between a wild-type egg chamber (anterior) and a *Delta* germline clone (posterior). The posterior (arrow) and anterior (arrowhead) pairs of polar cells of the wild-type anterior egg chamber are clearly visible with the polar cell specific marker A101 (blue).

(F) The expression of A101 (blue) and HTS (red) in a compound egg chamber containing one wild-type cyst (anterior) and two *Delta* mutant cysts. Polar cells form at each end of the wild-type cyst but cannot be detected between the mutant cysts, confirming that the polar cells (arrow) between a mutant cyst and the fused wild-type cyst are induced by the latter.

observed with other polar cell markers, such as *unpaired* mRNA, which is also first expressed in anterior cells at stage 1 and in posterior cells at stage 2 (Figure 3D).

The delayed development of the posterior polar cells is also reflected in later aspects of their behavior. The stalk remains associated with the polar cells until they round up and become clearly distinct from the epithelial follicle cells. This occurs earlier in the anterior polar

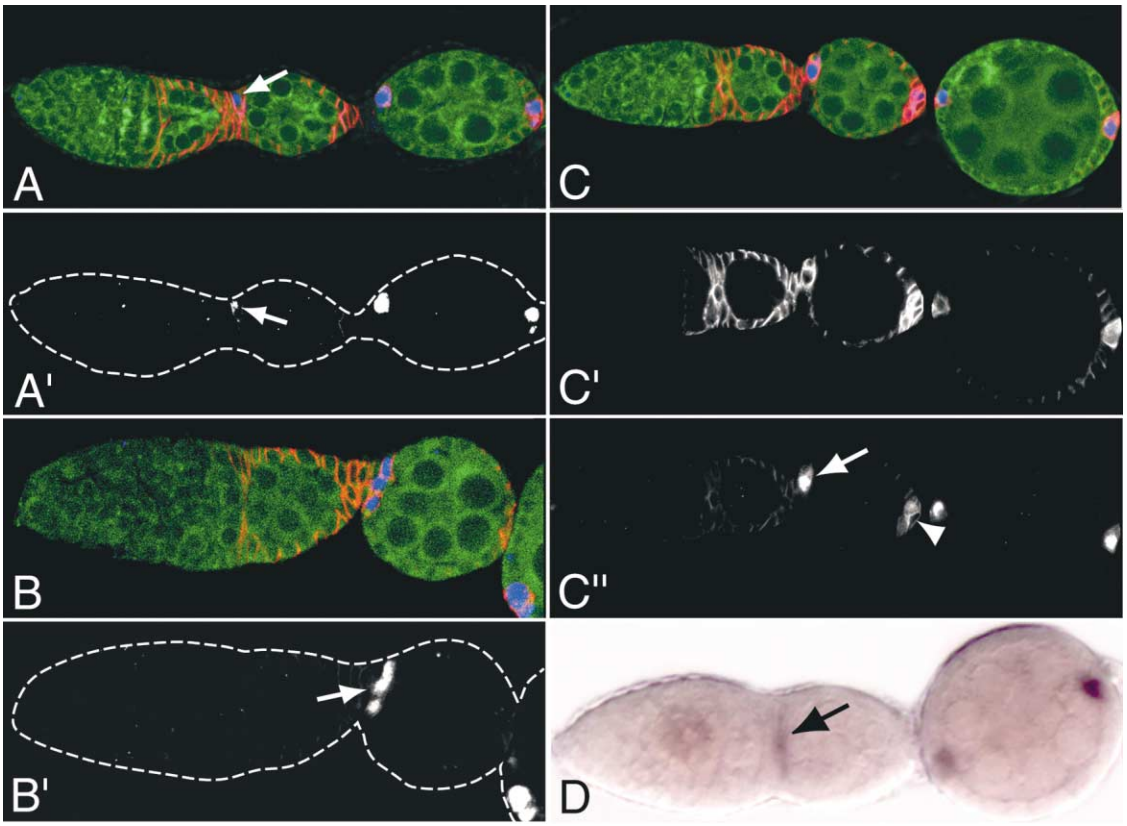

Figure 3. The Anterior and Posterior Polar Cells Differentiate Asynchronously

(A–C) Wild-type ovarioles expressing A101 (blue and A′, B′, and C″) and stained for FasIII (red, C′). The green channel shows the background autofluorescence to reveal the arrangement of cells in the germarium.

(A) A101 is first expressed in the anterior polar cells at stage 1 (arrow) as the cyst starts to bud off from the germarium but only appears in the posterior polar cells at stage 2.

(B) By the time the cyst has budded from the germarium, A101 is expressed in four or more anterior polar cells (three in this focal plane, arrow) but is still not expressed at the posterior. FasIII (red) is also enriched in the anterior polar cells at this stage.

(C–C″) The expression of FasIII (red and C′) becomes restricted to the two anterior polar cells at stage 2, which can also be distinguished by their round shape. By this stage, FasIII is still expressed at higher levels in three to four posterior follicle cells, but at stage 3 to 4 this expression becomes restricted to only two cells, which also have a round shape. A101 (blue and C″) is first expressed at low levels in the posterior polar cells at stage 2, when it is already expressed very strongly in the anterior polar cells (arrow). A101 is expressed at equally high levels in the anterior and posterior pairs of polar cells by stage 3 to 4.

(D) In situ hybridization to *unpaired* mRNA. *unpaired* is expressed in the anterior polar cells at stage 1 but only appears in the posterior polar cells at stage 2, similar to A101.

cells, which detach from the stalk during stages 2 to 3, whereas the posterior polar cells detach at stage 4 to 5 (Figures 1A and 2A).

The observation that there are initially four polar cells at the anterior of the egg chamber and none at the posterior but that there are always two polar cells at each end of the egg chamber in later stages raised the possibility that all polar cells are specified at the anterior and that two of them migrate to the posterior. To test this, we searched for polar cells between the poles of wild-type egg chambers during stages 1 to 2. In more than 100 egg chambers analyzed, we never found any polar cell more than one cell diameter from either the anterior or posterior pole, making it very unlikely that the anterior cells migrate to the posterior. This suggests that the polar cells differentiate independently at the anterior and posterior of the egg chamber and that the number of polar cells is then regulated, consistent with recent findings that excess anterior polar cells undergo apoptosis (Besse and Pret, 2003).

Posterior Polar Cells Are Not Required for Stalk Formation or Oocyte Positioning

The polar cells signal through the JAK/STAT pathway to induce the formation of the stalk that separates adjacent cysts (Baksa et al., 2002; McGregor et al., 2002). The stalk is initially flanked by the anterior polar cells of one cyst and the posterior polar cells of the adjacent younger cyst. To analyze the contribution that each pair of polar cells makes to stalk induction, we examined whether stalks form on either side of single *Delta* mutant cysts. Consistent with our previous observation that fusions always occur at the anterior of a mutant cyst, a stalk never forms at the anterior of a *Delta* germline clone (Figure 4A). This phenotype is fully penetrant whether the fused anterior cyst is mutant or wild-type (Figures

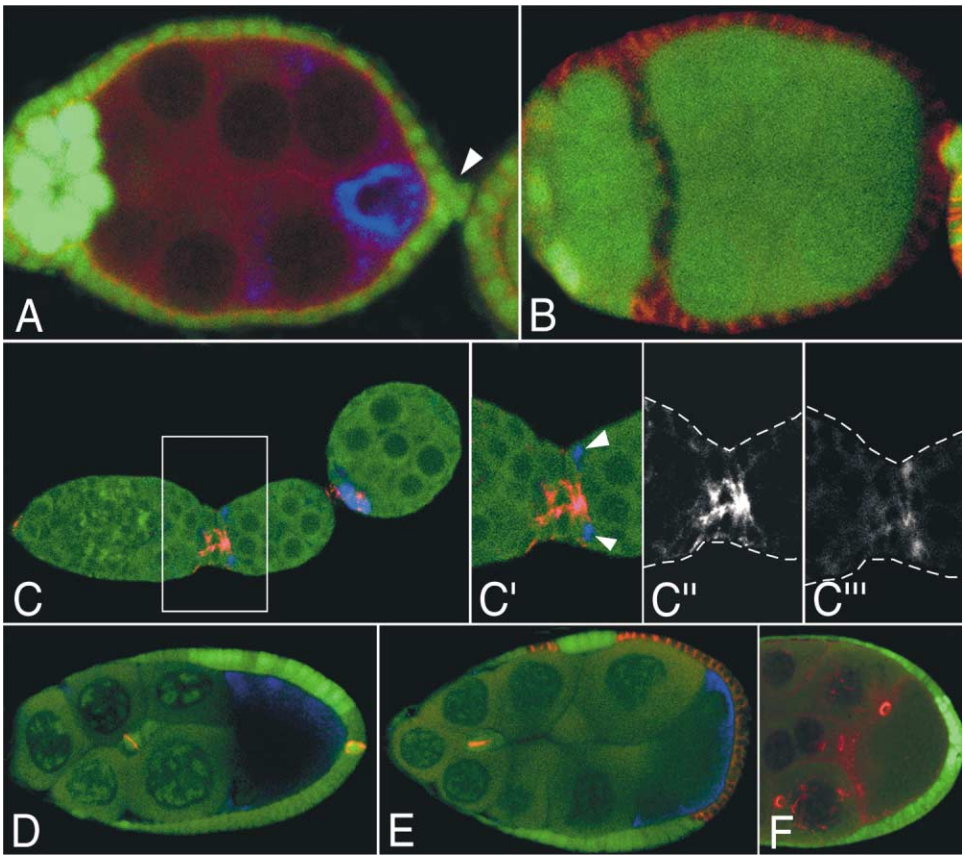

Figure 4. Posterior Polar Cells Are Not Required for Stalk Formation or Oocyte Positioning

(A) A *Delta* germline clone marked by the absence of nuclear GFP (green) and stained with anti-α-catenin (red) and anti-Orb (blue). A stalk has not formed on the anterior side of the mutant cyst, which fuses with the anterior wild-type egg chamber, but the posterior stalk has formed normally (arrow).

(B) A cyst entirely surrounded by a *Notch* follicle cell clone (marked by the absence of nuclear GFP in green and the upregulation of FasIII in red). This egg chamber lacks an anterior stalk and has fused with the anterior egg chamber, but it has a posterior stalk.

(C) Wild-type ovariole stained for Bigbrain (Bib) to mark the stalk (red, C″) and A101 (blue, C‴) to label the polar cells. The stalk begins to form at the anterior of the cyst in stage 1, immediately adjacent to the anterior polar cells (detail in C′, arrowheads). The positions of the cells in the germarium are revealed by the green autofluorescent signal.

(D and E) Stage 9 egg chambers stained for FasIII (red) and BicD (blue), which labels the oocyte.

(D) Wild-type.

(E) An egg chamber with a large posterior clone of *Notch* mutant follicle cells, which are marked by the loss of GFP (green) and the upregulation of FasIII (red). Although there are no posterior polar cells, the oocyte is correctly positioned at the posterior of the egg chamber.

(F) Detail of the posterior of an egg chamber containing a *Delta* germline clone (marked by the loss of GFP in green) that has been stained for A101 (blue) to mark the polar cells and HTS (red) to label the ring canals. The oocyte is positioned at the posterior of the cyst, as in the wild-type, even though the mutant egg chamber lacks posterior polar cells.

2C, 2E, and 2F). The stalk is always present at the posterior of the mutant cyst, however, provided that the adjacent older egg chamber is wild-type (Figure 4A, arrowhead). Similar phenotypes are caused by *Notch* mutant follicle cell clones. Anterior follicle cell clones of N^{55e11} that include the putative stalk/polar follicle cell precursors lack anterior polar cells and fail to form an anterior stalk. In contrast, follicle cell clones that cover the entire posterior of the egg chamber always have a posterior stalk, as long as one of the anterior polar cells of the older egg chamber is wild-type (Figure 4B; data not shown). These results demonstrate that the anterior polar cells induce the formation of the stalk, but the posterior polar cells play no role in this process.

We also analyzed the relative timing of stalk and polar cell differentiation, using an antibody against Bigbrain (Bib), which is the earliest known marker for the stalk, and A101 to label the polar cells (Larkin et al., 1996; Tworoger et al., 1999). The anterior polar cells differentiate just as the stalk begins to form (Figure 4C). Since these cells lie immediately adjacent and posterior to the incipient stalk and express the stalk inducer Unpaired (Figure 3D), they are present at the right place and time to induce stalk formation. In contrast, the posterior polar cells arise about 24 hr after the stalk has formed, since it had already been induced by the anterior polar cells of the preceding cyst two stages earlier.

It has recently been proposed that the posterior polar cells play a key role in positioning the oocyte, since these cells end up in the correct position at the posterior of the oocyte, and the oocyte is often misplaced when they are not specified due to a posterior *fringe* mutant

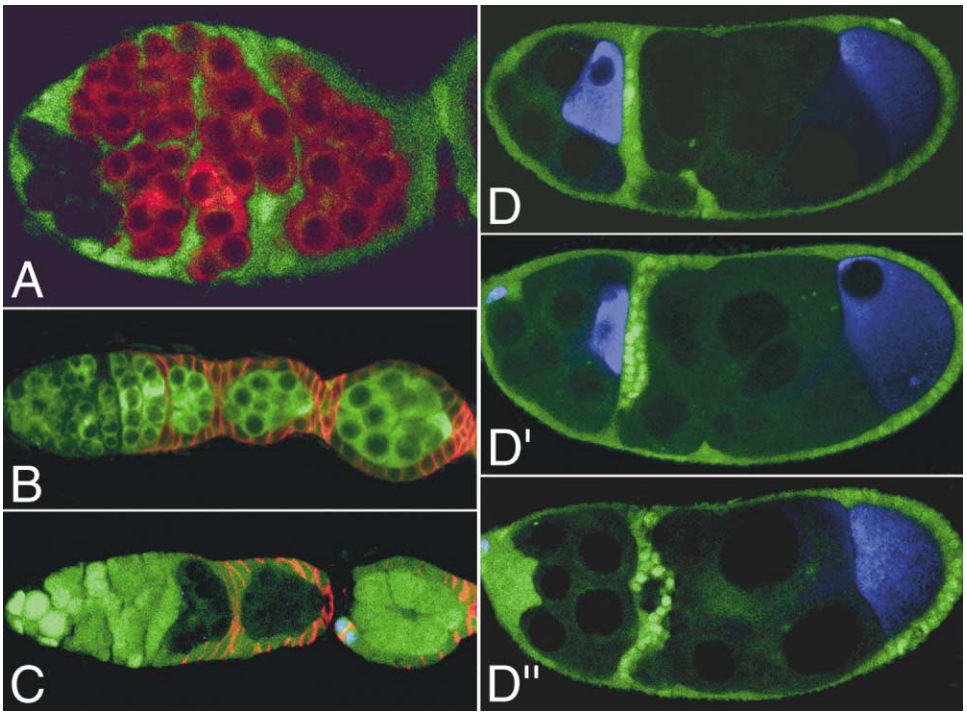

Figure 5. Delta Is Not Required for Follicle Cell Migration

(A) Germarium containing several *Delta* germline clones (marked by the absence of nuclear GFP in green) and stained for Orb (red), which marks the germ cells. The follicle cells migrate to completely surround the mutant cysts.

(B) Wild-type germarium stained for FasIII (red) and BicD (green).

(C) Germarium containing two consecutive *Delta* germline clones marked by the absence of nuclear GFP (green) and stained for A101 (blue) and FasIII (red). The follicle cells migrate normally to encapsulate the mutant cysts.

(D–D″) Three optical sections through a compound egg chamber containing two *Delta* mutant cysts, which have been stained for Orb (blue) to label the oocytes. The double layer of follicle cells between the two cysts is beginning to collapse and is interrupted by gaps (D′) and regions where the two epithelia have come apart (D″).

clone (Grammont and Irvine, 2002). Our results argue against a role for the posterior polar cells in this process. The oocyte is always localized (200/200) at the posterior of single *Delta* mutant germline cyst, even though these cysts never form posterior polar cells (Figures 4D and 4E). Furthermore, the oocyte is always correctly positioned in egg chambers that contain large posterior *Notch* follicle cell clones, which also lack posterior polar cells (Figure 4F). Thus, the posterior polar cells are dispensable for both stalk formation and oocyte positioning.

Delta Is Not Required for Follicle Cell Migration

It has been suggested that *Delta* germline clones and *Notch* follicle cell clones cause fusions between adjacent egg chambers because the follicle cells fail to migrate to encapsulate cysts in the germarium (López-Schier and St Johnston, 2001). We therefore examined the behavior of the follicle cells in germaria that contain *Delta* mutant germline clones. In all germaria analyzed, the follicle cells migrate normally to surround mutant cysts as they move from region 2b into region 3 (Figures 5A–5C), suggesting complete fusions must arise later in oogenesis. We therefore scored the frequency of fusions at different stages and observed that complete fusions occur mainly after stage 7 (data not shown). In earlier stages, there is usually a double-layered epithelium of follicle cells between the mutant cyst and the anterior wild-type cyst, even though these cysts are not separated by a stalk (Figures 2E and 2F). In contrast, most mutant cysts after stage 8 are completely fused to the adjacent anterior cyst, whether it is mutant or wild-type (Figures 1B, 2C, and 2C′). Consistent with this, we found several stage 7 to 8 mutant cysts where the double layer of follicle cells appears to be in the process of collapsing (Figures 5D, 5D′, and 5D″). These results suggest that the epithelial follicle cells form normal epithelia around the *Delta* mutant cyst and the adjacent wild-type cyst, but these are not stable in the absence of the stalk that normally separates them.

Delta Is Required to Position the Oocyte of the Adjacent Cyst to the Posterior

Although the oocyte of *Delta* mutant cysts is always correctly positioned at the posterior, the oocyte of the anterior adjacent wild-type cysts is mislocalized (Figure 1), indicating that Delta signaling from the older cyst is required for the anterior-posterior polarization of the younger one. To determine the cause of this phenotype, we examined the arrangement of germ cells in cysts anterior to *Delta* mutant cysts at various stages of oogenesis. These cysts flatten normally in region 2b of the germarium to form a disc with the oocyte in the center, but they fail to undergo the subsequent morphogenetic

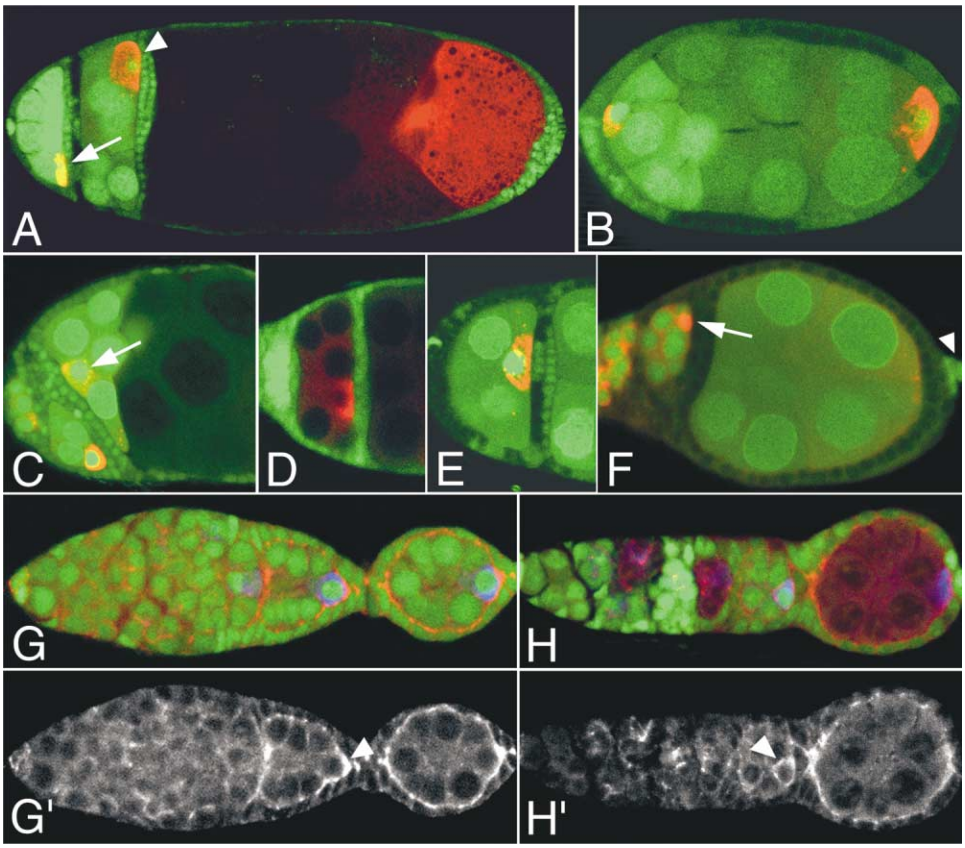

Figure 6. Delta Is Required for the Positioning of the Oocyte in the Adjacent Younger Cyst

(A–E) Compound egg chambers containing *Delta* germline (A, C, D) or follicle cell (A, B, E) clones marked by the absence of GFP (green) and stained for Orb (red) to mark the oocytes.

(A) A *Delta* germline mutant cyst partially fused to the anterior wild-type cyst, which has the oocyte at the corner of the flat posterior margin (arrowhead). This cyst is fused to the next cyst due to a *Delta* follicle cell clone and also has the oocyte mislocalized to the corner (arrow).

(B and C) Incomplete fusions produced by *Delta* follicle cell (B) or germline (C) clones; the oocyte of the anterior cyst is mislocalized to the anterior (C, arrow).

(D and E) In partial fusions caused by *Delta* germline (D) or follicle cell (E) clones, the bilayered epithelium that separates the two cysts is completely flat. The oocyte of the anterior cyst lies in the middle of the straight posterior border of the cyst that abuts this epithelium and fails to protrude into the follicle cell layer.

(F) A cyst surrounded by a *STAT92E*^{j6C8} follicle cell clone. The anterior stalk does not form, resulting in a fusion with the anterior cyst and the mispositioning of its oocyte (arrow). The stalk forms normally at the posterior (arrowhead).

(G) Wild-type ovariole stained for α-catenin (red, G′) and Orb (blue). The oocyte protrudes into the posterior follicle cell layer as the cyst rounds up on entering region 3 (arrowhead).

(G′) α-catenin becomes highly enriched in the area of contact between the oocyte and the follicle cells.

(H) Ovariole containing a *Delta* germline mutant cyst at stage 2. Although the wild-type cyst anterior to the mutant cyst has entered region 3, its oocyte does not protrude into the posterior follicle cell layer.

(H′) The oocyte of the wild-type cyst upregulates α-catenin as in wild-type (arrowhead), but α-catenin does not accumulate between the oocyte and these cells.

movement that positions the oocyte. Although the cyst loses the tight disc shape as it moves through region 3, it does not round up into a sphere, and the oocyte never protrudes into the posterior follicle cell layer (Figures 6A, 6D, and 6E). Instead, the posterior boundary between the cyst and the follicle cell epithelium remains straight throughout oogenesis, so that nearly all of the germ cells lie in equivalent positions along the A-P axis. The oocyte initially lies in the middle of this flat posterior side of the cyst (7/7 cysts before stage 4), but it usually moves to one corner of the flat boundary by the time the posterior mutant cyst reaches stage 5 to 6 (45/62 cysts, Figure 6A). The direction of this movement appears to be random, since there is no correlation between the corner that the oocyte occupies and the side of the egg chamber that contains the posterior polar cells (Figure 2C). This confirms that the latter play no role in oocyte positioning. Once the follicular epithelium between the two cysts breaks down, the oocyte moves to the anterior (16/16 cysts), where it remains throughout oogenesis (Figure 6C).

The results above indicate that the older cyst induces the positioning of the oocyte in the younger cyst, most probably via the stalk that connects them. It is also possible, however, that the stalk is not required for this process and that the older cyst or its anterior polar cells trigger the morphogenetic change required to position the oocyte. To distinguish between these possibilities, we examined cysts with *Delta* mutant follicle cell clones, which do not form a stalk but still form polar cells in

response to the Delta signal from the germline (López-Schier and St Johnston, 2001; Ruohola et al., 1991; I.L.T. and D.StJ., unpublished data). When the follicle cell clones result in partial fusions, they cause a similar phenotype to *Delta* germline clones. The anterior cyst fails to round up into a sphere and forms a straight posterior border with the follicular epithelium that separates it from the older cyst (Figures 6A and 6E). The oocyte fails to protrude into the follicle cell layer and usually moves to one corner of the cyst at early stages and then to the anterior when the follicular epithelia between the two cysts break down (Figures 6A, arrow, and 6B). To verify these results, we analyzed the position of the oocyte in compound egg chambers produced by follicle cell clones of two alleles of *STAT92E* (*STAT92E*^{j6C8} and *STAT92E*^{06346}), which cause a cell-autonomous loss-of-stalk cell fate, because the cells cannot respond to Unpaired signaling from the polar cells (McGregor et al., 2002). As expected, the oocyte of the anterior cyst is mislocalized when the posterior stalk is missing because the follicle cells between it and the adjacent posterior cyst are mutant for *STAT92E* (Figure 6F, arrow). Furthermore, STAT92E is only required in the stalk cells, since the oocyte is positioned correctly when all of the epithelial follicle cells and the polar cells are mutant, but the stalk cells are wild-type (posterior cyst in Figure 6F [arrowhead]). Thus, the positioning of the oocyte requires the posterior stalk.

The positioning of the oocyte requires the upregulation of the *D*E-cadherin adhesion complex in both the oocyte and the posterior follicle cells, which causes them to adhere to each other more strongly than to other cells (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998a). This can be visualized in wild-type ovaries by staining for components of the complex, such as α-catenin, which becomes concentrated at the boundary between the oocyte and the posterior follicle cells as the oocyte protrudes into the follicle cell layer (Figures 6G and 6G′, arrowhead). Components of the *D*E-Cadherin adhesion complex are still enriched in the oocyte of the cyst anterior to a *Delta* germline clone (Figure 6H′, arrowhead), but the posterior follicle cells no longer appear to express higher levels than the other cells (Figure 6H; data not shown). A similar phenotype is caused by *Delta* follicle cell clones (data not shown). Thus, stalk induction by the older cyst is required for both of the processes that mediate oocyte positioning: the rounding up of the cyst in region 3 and the preferential adhesion of the posterior follicle cells to the oocyte.

Discussion

It has previously been thought that the polar and terminal follicle cells at each end of the egg chamber are equivalent until around stage 5 to 6 of oogenesis, when Gurken signals from the oocyte to induce the adjacent cells to adopt a posterior fate (González-Reyes et al., 1995; Roth et al., 1995). Our results reveal, however, that there are a number of differences between the anterior and posterior pairs of polar cells when they differentiate during stages 1 to 2. First, these cells arise asynchronously, since the anterior polar cells appear about 12 hr before the posterior ones. Second, about four cells initially express polar cell markers at the anterior, whereas only two, or very occasionally three, cells express these markers at the posterior. Third, only the anterior polar cells are competent to induce the formation of the stalk, since normal stalks form at the posterior of a *Delta* mutant cysts, which lack posterior polar cells. Furthermore, posterior polar cells are not sufficient for stalk formation, as they are unable to induce a stalk when the older cyst lacks anterior polar cells.

It is unclear why posterior polar cells differentiate later than the anterior ones, since both depend on the same Delta signal from the germline. One possibility is that this difference occurs because the Delta signal itself is asymmetric. The oocyte has already reached the posterior by the time that this induction occurs, and it is therefore possible that Delta signals more strongly or earlier from the nurse cells at the anterior of the germline cyst than from the posterior oocyte. Alternatively, the asynchrony could reflect an intrinsic difference in the competence of the polar cell precursors to respond to Delta. Clonal analysis indicates that the precursors of the anterior polar cells of one cyst, the stalk cells, and the posterior polar cells of the adjacent younger cyst are closely related, indicating that they all arise from the group of polar/stalk precursors that migrate between the cysts in region 2b of the germarium (Margolis and Spradling, 1995; Tworoger et al., 1999). Thus, the posterior polar cell precursors migrate at the same time as those of the anterior polar cells of the adjacent older cyst, whereas the precursors of the anterior polar cells of the same cyst only arrive about 12 hr later, when the next cyst reaches region 2b. This means that posterior polar cell precursors are significantly older than their anterior counterparts, and this may reduce their competence to respond to the Delta signal, leading to a delay in their differentiation. One reason why this might occur is because these cells have already been exposed to Unpaired signaling from the anterior polar cells of the older cyst and have therefore started to differentiate as stalk, which may make it more difficult to induce them to switch into the polar cell pathway.

The difference in the timing of the differentiation of anterior and posterior polar cells probably accounts for all the other asymmetries between these two sets of otherwise identical cells. For example, the posterior polar cells may be unable to induce the stalk because, by the time they are specified, the polar/stalk cell precursors have lost their competence to respond to the inductive signal. Activation of the JAK/STAT pathway is both necessary and sufficient to induce polar/stalk precursors to adopt the stalk cell fate, and the ligand for this pathway, Unpaired, is expressed in both sets of polar cells (McGregor et al., 2002). The posterior polar cells only express Unpaired at stage 2, however, whereas the stalk is induced about a day earlier, when the cyst enters region 3 of the germarium. Although the expression of Unpaired by the posterior polar cells plays no role in stalk formation, it is not redundant because Unpaired also induces the epithelial follicle cells around the polar cells to adopt a terminal fate, and this is essential later in oogenesis to render these cells competent to respond to Gurken by becoming posterior. The delay in the differentiation of the posterior polar cells can also explain why only two, or very occasionally three, cells are initially specified at this end of the egg chamber, compared to the four or more cells that arise at the anterior, since

most of the stalk/polar precursors have already adopted the stalk fate by this stage, and there will therefore be many fewer uncommitted precursors that are still competent to become polar cells. Finally, this delay can account for the later differentiation of the posterior polar cells, which is reflected in the fact that they round up and detach from the stalk during stages 4 to 5, whereas the anterior polar cells do this during stages 2 to 3. Thus, all of the differences between the anterior and posterior polar cells are temporal, and the two pairs of cells are identical in terms of their differentiation and gene expression from stage 2 until stage 6, when Gurken signals to break this symmetry.

A Relay Model for A-P Axis Formation

The positioning of the oocyte at the posterior of the germline cyst generates the first anterior-posterior polarity in *Drosophila* development and ultimately leads to the formation of the A-P axis of the embryo. Our results indicate that the asymmetric induction of the polar cells plays a key role in generating this polarity and leads us to propose a model in which each cyst induces the positioning of the oocyte in the following younger cyst. (1) By the time a germline cyst reaches region 3 of the germarium, it has already positioned its oocyte to the posterior and is separated from the adjacent younger cyst in region 2b by a pool of uncommitted polar/stalk precursors. At this point, Delta signals from the germline cyst to activate Notch in the adjacent anterior polar/stalk precursors, thereby inducing them to develop as polar cells (Figure 7A). (2) These anterior polar cells turn on Unpaired and induce the more anterior polar/stalk precursors to differentiate as stalk cells (Figure 7B). (3) The stalk cells intercalate with each other and converge toward the middle of the ovariole to generate a two cell-wide stalk. This morphogenetic movement causes the younger anterior cyst to round up, as it is pulled into region 3. In parallel, the stalk somehow induces the upregulation of *D*E-cadherin in the follicle cells that contact the oocyte of the younger cyst, and they therefore adhere preferentially to this cell. This positions the oocyte by causing it to protrude into the follicle cell layer, which anchors it at the posterior as the cyst changes shape (Figure 7C). (4) By the time that the younger cyst has positioned its oocyte, it has entered region 3 of the germarium and activates Delta signaling. This then induces polar cell fate in the polar/stalk precursors that have migrated to cover its anterior, and the cycle begins again. It is only at this stage that polar cells differentiate at the posterior of the older cyst, which has now exited the germarium and is at stage 2 (Figure 7D).

The oocyte is therefore positioned by a relay mechanism that involves a series of posterior to anterior inductions. The older cyst induces the anterior polar cells, the anterior polar cells induce the stalk, and the stalk induces the positioning of the oocyte of the younger anterior cyst. Each ovariole functions as a production line for new egg chambers, since the germline stem cells lie at one end and divide to provide a constant source of new cysts. This relay can therefore be repeated over and over again to position the oocyte at the posterior of each new cyst that passes through the germarium. In this way, anterior-posterior polarity is transmitted from one cyst to the next, to define the anterior-posterior axis of each egg chamber. This type of relay represents a simple mechanism for propagating a repeated asymmetric pattern.

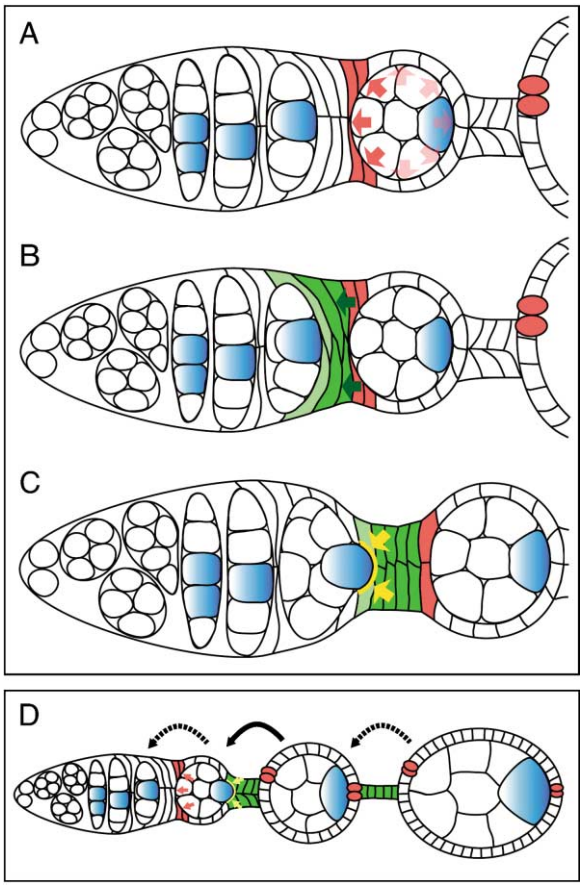

Figure 7. A Relay Model for Oocyte Positioning

(A) As a germline cyst buds from the germarium, it signals through the Delta/Notch pathway (arrows) to induce the formation of the anterior polar cells (red).

(B) The anterior polar cells express Unpaired, which activates the JAK/STAT pathway the adjacent stalk/polar cell precursors to induce them to become stalk (green).

(C) The stalk induces the positioning of the oocyte in the adjacent younger cyst by inducing the latter to round up, and the follicle cells that contact the oocyte to upregulate the *D*E-cadherin adhesion complex (yellow). The resulting increase in adhesion between these follicle cells and the oocyte causes the latter to protrude into the follicle cell layer as the cyst changes shape, which anchors it at the posterior.

(D) An overview of the steps in the relay to show how each cyst induces the positioning of the oocyte in the next cyst. As the oocyte is being positioned (yellow), a new round of Delta signaling begins in that cyst by the induction of anterior polar cells (red).

Posterior Polar Cells Are Not Required for Oocyte Positioning

One open question that remains is the identity of the cells that preferentially adhere to the oocyte to anchor it at the posterior. Although it has been previously proposed that the posterior polar cells fulfill this function (Grammont and Irvine, 2002), several pieces of evidence

demonstrate that they are not required for oocyte positioning. (1) The oocyte is always correctly localized at the posterior of *Delta* mutant cysts, which lack posterior polar cells. Large *Notch* mutant follicle cell clones at the posterior of the egg chamber also block posterior polar cell specification but have no effect on oocyte positioning. (2) The oocyte always becomes mislocalized in the wild-type cysts anterior to a *Delta* mutant cyst, even though the posterior polar cells are present, indicating that these cells are not sufficient for oocyte positioning. In fact, the oocyte shows no preference for contact with the posterior polar cells, when it moves to the side of the cyst. (3) The oocyte is positioned as it enters region 3 of the germarium, while the posterior polar cells are only specified a day later at stage 2. (4) Electron micrographs reveal that the oocyte contacts about six follicle cells as it protrudes from the posterior of the cyst on entering region 3, whereas there are only two polar cells (Koch and King, 1969).

Since the posterior polar cells do not position the oocyte, some other cells must fulfill this function. One possibility is that the stalk induces the epithelial follicle cells at the posterior of the cyst to upregulate *D*E-cadherin and adhere to the oocyte. Alternatively, the stalk cells could adhere to the oocyte directly. This would be consistent with the fact that all stalk cells upregulate *D*E-cadherin as the stalk forms and removes the requirement for a third induction from the stalk to the epithelia cells. Furthermore, such a direct contact would help to explain why the formation of the stalk causes the anterior cyst to round up. The cells that eventually become the posterior polar cells may also participate in this adhesion, and then switch to the polar fate at stage 2, because they remain in contact with the oocyte and are therefore exposed to the next round of Delta signaling.

A Different Cue Must Polarize the First Cyst in Each Egg Chamber

The relay between cysts that we describe above represents the most upstream event in *Drosophila* axis formation that has been discovered so far and can account for the origin of anterior-posterior polarity in the vast majority of egg chambers. It cannot explain, however, how the first cyst in each ovariole is polarized. This leads to the prediction that other cells must fulfill the function of the posterior stalk in positioning the oocyte of this first cyst, and good candidates would be the basal stalk cells of the pupal ovariole. It is worth noting that the polarization of each cyst does not require that the previous cyst has correctly positioned its oocyte, since wild-type cysts anterior to a *Delta* mutant cyst induce oocyte positioning in the next cyst, even though their own oocyte is misplaced. Thus, the polarization of the first egg chamber in each ovariole is not necessary for the polarity of all subsequent egg chambers.

Experimental Procedures

Fly Stocks

In this work we used the following stocks: w^{1118} (Lindsley and Zimm, 1992), FRT82B Dl^{M1}/*TM3* (de Celis et al., 1991), and N^{55e11} FRT101/*FM7* (Couso and Martinez Arias, 1994). FRT82B $STAT92E^{06346}$/*TM3* and $STAT92E^{16C8}$/*TM3* were kindly provided by Doug Harrison. The polar cells were marked with the A101 (Clark et al., 1994) and PZ80 (Karpen and Spradling, 1992) enhancer traplines. The FRT82B GFP chromosome was provided by Stefan Luschnig (Luschnig et al., 2000) and GFP FRT101 by Alfonso Martinez-Arias. To use the A101 enhancer trap line, we recombined it into the FRT82B Dl^{M1} chromosome by meiotic recombination to generate FRT82B A101 Dl^{M1}/*TM6C* flies.

Generation of Clones

Mutant clones were generated by mitotic recombination using the FRT-FLP technique (Xu and Rubin, 1993) by heat shocking FRT mutant/FRT GFP third instar larvae for 2 hr at 37°C for 3 consecutive days. Flies were kept at 25°C and dissected 2 to 5 days after heat shock. The mutant clones were marked by the loss of nuclear GFP expression (Davis et al., 1995). To generate clones in females that carry the PZ80 enhancer trap line, PZ80/*CyO* flies were crossed to w-;FRT82B Dl^{M1}/*TM3*. PZ80/+;FRT82B Dl^{M1}/+ males were then crossed to *hsflp22*;FRT82B GFP females to generate females of the genotype hsflp22/+;PZ80/+;FRT82B Dl^{M1}/FRT82B GFP.

Staining Procedures

Antibody stainings, in situ hybridizations, and ovary dissections were performed according to standard procedures. Antibodies were used at the following concentrations: anti-Orb (orb 6H4 and orb 4H8 DSHB), 1/400 (Lantz et al., 1994); anti-BicD, 1/1000 (Wharton and Struhl, 1989); anti-FasIII, 1/20 (Patel et al., 1987); anti-Myosin VI (3C7), 1/10 (Kellerman and Miller, 1992); anti-Bigbrain, 1/1000 (Doherty et al., 1997); anti-EYA (eva 10H6 DSHB), 1/300 (Bonini et al., 1993); anti-HTS (hts RC), 1/250 (Robinson et al., 1994); anti-βgal, 1/1000 (Cappel); anti-αCAT1, 1/100 (Oda et al., 1993); anti-DIG:AP, 1/2000 (Roche); and anti-Stau, 1/100 (St Johnston et al., 1991). Secondary antibodies conjugated with Cy5, Texas-Red, and FITC were used at 1/200 (Jackson Laboratories). The *unpaired* RNA antisense probe was kindly provided by James Castelli-Gair Hombria and used with a 1/10,000 dilution. Confocal images were collected using a BioRad MRC1024 scanning confocal microscope. Nomarski micrographs were captured using a Spot Camera (Diagnostic Instruments Inc.) on a Zeiss microscope.

Acknowledgments

We thank D. Harrison, J. Castelli-Gair Hombria, A. Martinez-Arias, S. Luschnig, K. Miller, H. Oda, Y.N. Yang, R. Wharton, the Developmental Studies Hybridoma Bank (University of Iowa), and the Bloomington Stock Center for fly stocks and reagents. I. Palacios, T.P. Munro, and A. Djiane provided useful comments on this manuscript. This work was supported by a Wellcome Trust Programme Grant (D.StJ.) and the Fundação de Sousas (I.L.T.).

Received: April 15, 2003
Revised: July 31, 2003
Accepted: August 5, 2003
Published: October 6, 2003

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